DETECTION OF *Macrobrachium rosenbergii* Nodavirus (*Mr*NV) AND EXTRA SMALL VIRUS (XSV) DISEASES ON GIANT FRESHWATER PRAWN, *Macrobrachium rosenbergii* AT SAMAS, YOGYAKARTA

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ABSTRACT

Mass mortality of giant freshwater prawn (*Macrobrachium rosenbergii* de Man) in grow-out farmers occurred in early February 2012 at Instalation Coastal of Aquaculture Samas, Bantul, D.I. Yogyakarta. The clinical sign of shrimp was whitish coloration on abdominal and tail muscle. The sympton was the same as in other cases of mortality caused by prawn *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and Extra Small Virus (XSV). Prawn samples were diagnosed by standard protocols Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) using specific primers and histopathology analysis. The result showed that all samples indicated positive 13/15 the *Mr*NV and 5/15 positive XSV, and there were 4/15 positive samples both (*Mr*NV and XSV). Analysis of histopathology showed that damaged muscle was indicated by the presentation of necrotic tissues with nuclear pyknosis or degeneration of muscle in infected tissues. Based on diagnosis by RT-PCR and histopathological, mass mortality of the giant freshwater prawn in Indonesia is determined to be caused by "white muscle disease (WMD)/white tail disease (WTD)".

KEYWORDS: freshwater prawn, *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and Extra Small Virus (XSV), RT-PCR, histopathology

INTRODUCTION

The giant freshwater prawn (*Macrobrachium rosenbergii*) is an economically potential commodity due to its good market share. Prawn farming techniques have been developed and performed by farmers and entrepreneurs widely. However, along with the development of good farming techniques there have been always problems of emerging disease. Some diseases could be caused by protozoa such as *Epistylis* sp., *Zoothamium* sp., and *Vorticela* sp., while bacterial diseases could be caused by *Aeromonas* sp., *Psedomonas* sp., *Enterobacter* sp., and *Vibrio* sp. Others such as black spot disease, black gills disease, and *Idiopathic muscle necrosis* (IMN) or white muscle disease, and another disease caused by fungus such as *Fusarium* sp. These kinds of diseases have been reported to be infecting prawn farming especially in larval stadia (Supriyadi *et al*., 2001). But viral infectious disease is a major constraint in prawn farming and could cause high mortality and economic losses as in the case of *White Spot Syndrome Virus* (WSSV**)** infection in tiger prawn (*Penaeus monodon*) and whiteleg prawn (*Litopenaeus vannamei*).

Viral infections in prawns have not been widely identified scientifically in Indonesia. Viral diseases could occur at any time and

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could be caused by environment changes, high intensive system of prawn farming or free trading causing unproperly applied quarantine rules which made some new diseases can spread easily. A new viral disease which caused 30%-40% mortality of giant freshwater have occurred in early 2012 at Samas Yogyakarta. The major symptom of infected prawns are whitening muscle in the tail and abdominal, or known as White Tail Disease (WTD).

According to several reports, the WTD is caused by *Macrobrachium rosenbergii* nodavirus (*Mr*NV) or Extra Small Virus (XSV). The virus has been known could infect the prawn both at post-larva (PL) and adult stage (Arcier *et al*., 1999; Qian *et al*., 2003; Widada *et al*., 2003; Hameed *et al*., 2004a; Hsieh *et al*., 2006; Yonganandhan *et al*., 2006; Wang *et al*., 2008). Detection of WTD could be conducted by several methods such as histology and electron microscope. Sandwich enzyme linked immunosorbent assay methods (S-ELISA) based on antigen-antibody interaction (Romestand & Bonami, 2003). Genome-based detection by dot-blot technique, in situ hybridization and *Reverse Transcriptase Polymerase Chain Reaction*/RT-PCR (Widada *et al*., 2003; Hameed *et al*., 2004a; Shekhar *et al*., 2006; Yonganandhan *et al*., 2006; Hsieh *et al*., 2006; Behera *et al*., 2011). Detection with loop-mediated isothermal amplification method or LAMP has been known (Pillai *et al*., 2006). Now there is XSV specific detection based on genome analysis (Widada *et al*., 2004). Simultaneous detection in one tube for WTD (*Mr*NV-XSV) has also been tried (Yonganandhan *et al*., 2005; Tripathy *et al*., 2006). Qualitative detection could be conducted by using Tagman probe for Real Time or Quantitative Polymerase Chain Reaction (q-PCR) (Zhang *et al*., 2006).

Based on all informations and studies of WTD, the purpose of this research is to confirm the diagnosis on the mortality of giant freshwater prawn with WTD symptoms at Instalation Coastal of Aquaculture Samas-Bantul D.I. Yogyakarta by using histopathology and RT-PCR method.

MATERIALS AND METHODS

Samples of infected giant freshwater prawn (pleopod, muscle, tail, and gills) were taken in the middle of February 2012 from Instalation Coastal of Aquaculture Samas, Bantul, D.I. Yogyakarta and were kept in RNA later solution. Meanwhile, the whole specimen of giant freshwater prawn were kept in Davidson's solution for 24 hours before being transfered to 70% Ethanol for histophatology analysis. All the samples were recorded for RT-PCR analysis in Fish Health Laboratory at the Center Research for Aquaculture in Jakarta, while histopathology analysis was conducted in Tuscon Arizona USA.

RNA Extraction

Extraction of total RNA was conducted by using TRI Reagent Kit. Tissue samples of 50-100 mg were placed on 1.5 mL sterile microtube. Those samples were air-dried before added 1 mL of lysis solution. Samples were incubated at room temperature (25°C) for 5 minutes, then centrifuged (High Speed Refrigerated Micro Centrifuge XT-160) at 12,000 rpm for 10 minutes at 4°C. Supernatant was transferred to a new microtube and added 200 mL Chloroform, then recentrifuged at 12,000 rpm for 10 minutes at 4°C. The clear part (aqueous phase) was then transferred to a new microtube. Furthermore, a total of 500 mL Isopropanol was added into the tubes and then vortexed for 5-10 seconds before being incubated at room temperature for 5-10 minutes. The suspensions were centrifuged at 12,000 rpm for 10 minutes at 4°C and supernatant were discarded, then 75% ethanol was added to wash the RNA pellet before being centrifuged again at 7,500 rpm for 5 minutes at 4°C. Ethanol was removed and the pellet was air-dried. The pellet was resuspended by 100-200 mL ddH_2O addition then the density was measured by NanoDrop. Result of the measurements ranges between 142–825 ng/µL with purity level of 1.83-1.95 at 260/280 nm wavelength. RNA was then diluted up to 100-200 ng/µL before amplification process.

Reverse Transcriptase-Polymerase Chain Reaction

Moleculer analysis was used to detect the existence of WTD caused by *Mr*NV and XSV on infected samples, RNA-2 specific primer for MrNV have 425 bp of target molecular weight (Hameed *et al*., 2004a), while XS1/XS5 specific primer for XSV have 500 bp of target molecular weight (Widada *et al*., 2004). The sequence of specific primer is shown in Table 1.

AccessQuick Master Mix was used to amplify or reproduce DNA/c-DNA targets. AccessQuick Promega 2x Master Mix (Tfl DNA poly-

Table 1. The specific primers used to detect WTD (*Mr*NV and XSV) of giant freshwater prawn infected samples

Pair	Name	Size (bp)	Primers sequences 5' ~ 3'	Reference
RNA-2	*Mr* NV2aF *Mr* NV2aR	425	gCg TTA TAg Atg gCA CAA gg AgC TgT gAA ACT TCC ACT gg	Hameed *et al*. (2004a) Gen Bank AY222840
XS1/XS5	XS1-R XS5-F	500	ggA gAA CCA TgA gAT CAC g CTg CTC ATT ACT gTT Cgg AgT C	Widada *et al*. (2004) Gen Bank AY247793

merase, dNTPs, magnesium sulfate, and reaction buffer) and AMV RT was added separately to each reaction. Amplification reaction of total 20 mL, each used 1 mL Reverse and forward primer (18 pmol). RT-PCR was performed in RT cycling conditions of 52°C for 30 minutes and 95°C for 2 minutes (to convert RNA to c-DNA), followed by 30 cycles at 94°C for 40 seconds, 55°C for 40 seconds, 68°C for 60 seconds and at 68°C for 10 minutes. Analysis of RT-PCR products on a 1.5% gel electrophoresis in 1x TAE buffer and stained with ethidium bromide, the target product was measured using a 100 bp DNA ladder/marker under UV-Transilluminator.

Histopathology Analysis

Infected tissue organ (muscle) were fixed in Davidson's solution for 24 hours then transferred for refixation process in 70% ethanol solution for futher histopathologycal analysis. Naturally infected tissue samples of giant freshwater prawn were analyzed at the Department of Veterinary Science and Microbiology - The University of Arizona, Tuscon, AZ, USA with case No. 12-152, 12 August 2012.

RESULT AND DISCUSSION

The mortality of giant freshwater prawn broodstock strain G-macro and Synthetic, was found in Instalation Coastal of Aquaculture Samas, Bantul, D.I. Yogyakarta in February 2012. Prawn were cultured using traditional farming systems in concrete ponds in area of 250 m^2 with density of 3-5 seed/m^2. Post-larvae source cames from Sukamandi, West Java. Mortality occurred after the prawn was kept for 2.5 months, and mortality rate reached 30%-40% (Figure 1).

The symptom is whitish discoloration and opacity of the abdominal and tail muscles of the prawn which is known as White Tail Disease or WTD (Figures 2a and 2b). According to the farmers, clinical symptom of infected prawns has existed for sometime, but there is no definitive explanation of the cause. The symptom is similar to giant freshwater prawn mortality cases found in France, India, Thailand, and China, which is caused by *Mr*NV and XSV (Arcier *et al*., 1999; Widada *et al*., 2003; Hameed *et al*., 2004a; Hsieh *et al*., 2006; Yoganandhan *et al*., 2006; Wang *et al*., 2008).

Figure 1. Conditions prawn ponds and infected giant freshwater prawn at Instalation Coastal of Aquaculture Samas, Bantul, D.I. Yogyakarta

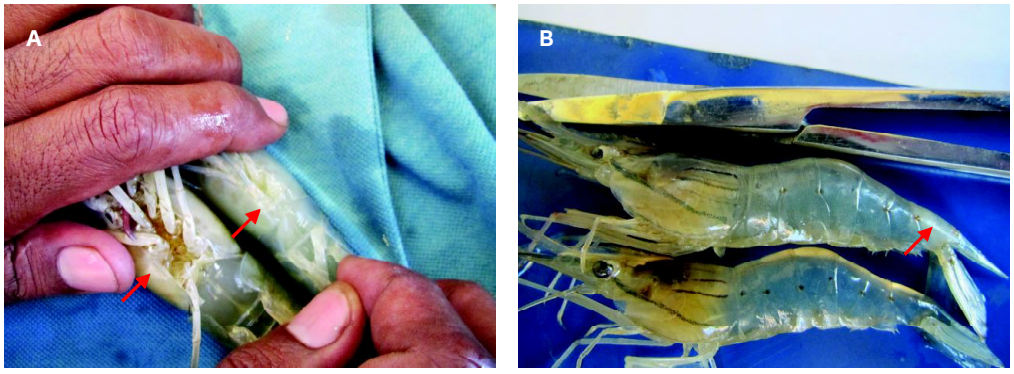


Figure 2. Gross signs of giant freshwater prawn infected with *Mr*NV, showing whitish discoloration and opacity of the abdominal (A) and (B) tail muscles (arrows) in Instalation Coastal of Aquaculture Samas, Bantul, D.I. Yogyakarta

According to Arcier *et al*. (1999), the case of WTD at post-larvae (PL) stage, the whitish coloration is hardly visible. Mortality will occur 5 days after the sight of early symptom or the existence of whitish coloration on its body.

The RT-PCR amplification with specific primer *Mr*NV and XSV (Hameed *et al*., 2004a; Widada *et al*., 2003), has shown that there is an indication of WTD symptom on tissue samples of infected giant freshwater prawn which undergo mass mortality. Out of 15 samples (13/15) are positive *Mr*NV. Meanwhile, for XSV diagnosis, 5 out of 15 samples are infected with XSV. Furthermore, there are 4 samples (4/15) which are positively infected by both *Mr*NV and XSV (Table 2).

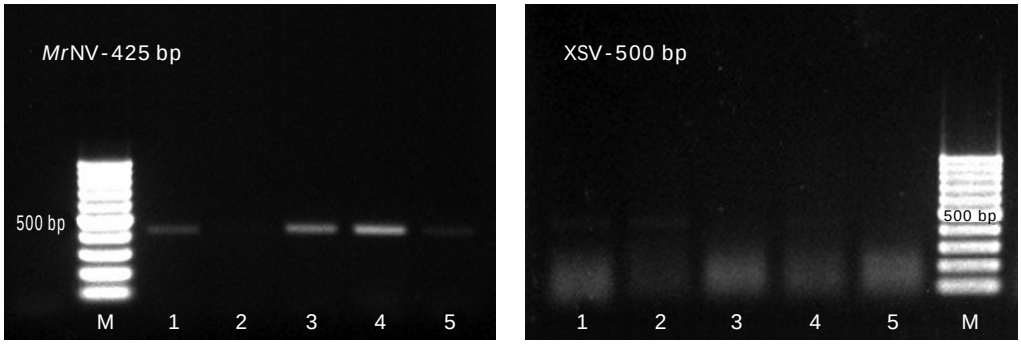
Almost at the same periode of time, mortality also found in other area such as Sukamandi, Pangandaran, and Sukabumi with WTD symptom but with lower rate of mortality. It seems that the cause of mortality is the same (data is not shown). It has been known for quite some time that WTD is the main obstacle on giant freshwater prawn farming on hatchery or grow up ponds. This is similar with WSSV infection case on Tiger prawn farming, which has not been resolved until this time. WTD was firstly identified in the hatchery of giant freshwater prawn at Pointe Noire, France. For periods of 10 years (1985-1994) those hatchery undergo mass mortality up to 5%-90% cumulative mortality.

RT-PCR diagnosis could be seen from the amplification result on gel electrophoresis, where each type of virus was shown in different target of molecular weight. The samples with 425 bp molecular weight means positive result for *Mr*NV and 500 bp for XSV (Figure 3).

Table 2. Diagnostic amplification RT-PCR *Mr*NV and XSV results of natural infection freshwater prawn from Instalation Coastal of Aquaculture Samas, Bantul, D.I. Yogyakarta

Samples freshwater prawn	Viruses detection	
	***Mr* NV**	**XSV**
1	**Positive**	**Positive**
2	Positive	Negative
3	Positive	Negative
4	Positive	Negative
5	Positive	Negative
6	Positive	Negative
7	Positive	Negative
8	Positive	Negative
9	Positive	Negative
10	**Positive**	**Positive**
11	Negative	Positive
12	**Positive**	**Positive**
13	Positive	Negative
14	**Positive**	**Positive**
15	Negative	Negative

Both types of primer are chosen due to its sensitivity level especially for *Mr*NV primer that could detect up to smallest concentration of 0.25 fg, while the XSV primer could detect on the level of 25 fg (Hammed *et al*., 2004a). WTD cases caused by *Mr*NV and XSV are commonly



Macrobrachium rosenbergii NodaVirus (425 bp) Extra Small Virus (500 bp)

Figure 3. Amplification product of *Mr*NV and XSV RT-PCR**,** in natural infection of giant freshwater prawn from Instalation Coastal of Aquaculture Samas, Bantul, D.I. Yogyakarta, 1-5 = samples, M = DNA ladder of 100 bp

found concurrently on mortality case of giant freshwater prawn. But, on specific case, WTD could also become the cause of XSV or *Mr*NV infection only, which is the case of Samas, Yogyakarta. This is still under research to find out the role of each cause of WTD.

There are studies on the role of XSV on WTD case, which remains unclear up to now. There are 3 hypothesis on the role of XSV: first, XSV is not a virus developing in giant freshwater prawn but infecting the prawn food such as *Artemia*, though the attemp to extract XSV directly from *Artemia* were unsuccessful. Second, XSV is an autonomous virus of giant freshwater prawn and third, XSV could be a helper virus for *Mr*NV. Since XSV is always found assosiated with the larger virus (nodavirus) and located in muscle and connective cell of infected prawns, it could be an autonomous virus, a helper-type virus, or satellite-like virus (Qian *et al*., 2003; Yonganandhan *et al*., 2006).

*Mr*NV and XSV have been found to be able to infect seawater prawn (*Penaeus monodon* and *P. indicus*) at post larvae stage in hatchery, where the symptoms are the same with the one infecting giant freshwater prawn and generated positive result by amplification using RT-PCR analysis (Ravi *et al*., 2009). More than that, *Artemia* as the prawn natural food could also be infected with *Mr*NV and XSV. Artificial infection by oral administration to the prawn, can cause 100% mortality (Sudhakaran *et al*., 2006).

Histopathology analysis of mass mortality in Samas, Yogyakarta can be seen from photomicrographs of infected muscle tissues. Damaged muscle was indicated by the presentation of necrotic tissues with nuclear pyknosis or degeneration of muscle in infected tissues (Figure 4B), as a comparison (Figure 4A) is normal muscle tissue of giant freshwater prawn and other changes were hemocytic infiltration at gill infected tissue (Figure 4C) and inclusion bodies-haemocytic infiltration in hepatopancreas (Figure 4D). The description of damaging tissue is similar with the case in Taiwan such as the present of inclusion bodies in infected muscle tissue (Arcier *et al*., 1999; Hsieh *et al*., 2006).

On the use of artificial infection, *Mr*NV and XSV could infect the whole organ of the prawn (Post-larvae stage, gill, head muscle, stomach, intestine, heart, hemolymph, pleopod, ovaries, and tail muscle), but the virus was not found in hepatopancreas and eyestalk (Hameed *et al*., 2004b).

In observation with Transmition Electron Microscope (TEM), it can be seen that morphologically *Mr*NV is a very small particle virus with the shape of icosahedral - non enveloped size in 25-30 nm which is located in the cytoplasm of connective tissues at all organs (Arcier *et al*., 1999; Romestand & Bonami, 2003; Qian *et al*., 2003). The shape and diameter size of XSV is smaller (between 14-16 nm), could be identified using TEM and it is usually mixed with the *Mr*NV (Qian *et al*., 2003).

This is the first report of White Tail Disease caused by *Mr*NV and XSV in Indonesia. Earlier, *Mr*NV, and XSV has been found in several countries with giant freshwater prawn farming such as India (Widada *et al*., 2003; Hameed *et al*.,

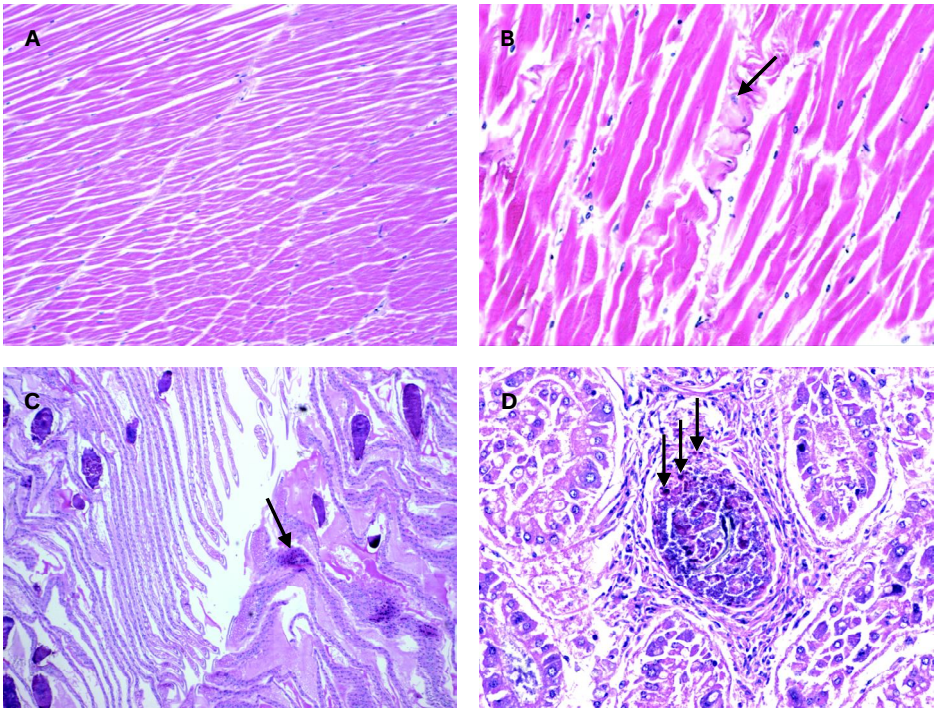


Figure 4. Photomicrograph-histopatology of organ and muscle from giant freshwater prawn infected with WTD (A) normal muscle tissue; (B) infected muscle tissue with nuclear pyknosis (arrow); (C) gill infected tissue with hemocytic infiltration, and (D) hepatopancreas with inclusion bodies and haemocytic infiltration (arrow)

2004a), Taiwan (Tung *et al*., 1999, Hsieh *et al*., 2006; Wang *et al*., 2008), China (Qian *et al*., 2003; Zhang *et al*., 2006), France (Arcier *et al*., 1999; Bonami *et al*., 2005), and Thailand (Yonganandhan *et al*., 2006).

Since this virus infects the ovarium, it is important to look for possible vertical infection from broodstock to its larvae. Hence, it is highly advised to be carefull when starting culture of giant freshwater prawn. It is recommended to use only broodstock which are free from both viruses by using RT-PCR technique. It is advised to use hemolymp or pleopod for non lethal samples since pleopod is the target organ for the detection of both viruses (Hameed *et al*., 2004b).

CONCLUSION

WTD was detected in giant freshwater prawn farming at Instalation Coastal of Aquaculture Samas, Bantul, D.I. Yogyakarta by using RT-PCR analysis and hispathology. Analysis showed that 13/15 and 5/15 of the samples indicated with positive infection to *Mr*NV and XSV respectively and there are 4/15 (four) positive samples both (*Mr*NV and XSV). It is important to do selection of giant freshwater prawn broodstock or post-larvae which are free of *Mr*NV and XSV. And also it is needed to do sequencing of nucleotide to identify *Mr*NV and XSV and compared it with data in Gene-bank.

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